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Nutrient Limitation and Adaptation of Microbial Populations to Chemical Transformations

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Using field-collected periphyton and bacterial isolates, we determined adaptation lag periods for microbial transformation of *p*-cresol. Lag periods were longer for periphyton samples collected from field sites that were low in dissolved inorganic nitrogen and phosphorus. Moreover, lag periods decreased in samples amended with N or P. Our data suggest that adaptation lag periods for microbial transformation of low concentrations of chemicals may correlate with limiting nutrient concentrations, and this correlation may provide a basis for predictive mathematical modeling of lag periods.

Considerable research continues to focus on the development of quantitative models for predicting microbial transformation rates of toxic chemicals that pollute aquatic and terrestrial ecosystems. However, microbial adaptation lag periods present significant problems with regard to modeling transformation rates. Few studies have examined the dynamics of adaptation, especially with respect to environmental factors that may influence adaptation lag periods. By definition, an adaptation lag period typically denotes a length of time during the initial exposure of microorganisms to a chemical in which the transformation rates of the chemical are too slow to be measurable or are significantly less than subsequent rates. Although their effects are apparent, the mechanisms involved in producing such lag periods are still unclear.

Because environmental studies relating to adaptation are typically done by using diverse and complex microbial communities, it frequently is not possible to conclusively state, based on the available data, what caused an observed lag period. Lag periods usually are attributed to changes in the relative numbers of organisms that are capable of transforming a chemical to organisms that are incapable of transformation, a phenomenon in which there is a concentration threshold for the substrate below which adaptation will not occur (1, 2, 17).

Transformation lag periods for naturally occurring and xenobiotic pollutants may be influenced by the presence and concentrations of additional substrates, vitamins, cofactors, essential elemental nutrients, such as N and P, and metabolic inhibitors and by a host of other chemical conditions that govern the metabolic activities of the innumerable species and strains of organisms in aquatic and terrestrial environments. Furthermore, because any combination of such factors may either restrict or promote transformation of a compound, what changes the lag period for one field site or subsample thereof may not have the same effect on other ecosytems or even on the same ecosystem tested at a different time.

However, it may be possible to determine that a few factors most frequently influence transformation lag periods. Hopefully, these factors involve parameters that are typically available in water quality data banks and are readily accessible to mathematical modelers. Paramount among these factors may be the effect of elemental nutrients, such as N and P, which commonly limit microbial activities in aquatic systems, and the diauxic effects of easily utilizable substrates, such as low-molecular-weight carbohydrates. With regard to the latter possibility, various substrates have been shown to cause lag periods for phenol transformation in pure cultures (14).

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MATERIALS AND METHODS

Field sites. Five freshwater field sites were sampled for periphyton by colonizing Teflon strips (7). These sites included two small ponds (Chalet Pond and Two-Boat Pond near Whitehall, Ga.), two river sites (the Middle and North Oconee Rivers near Athens, Ga.), and the Okefenokee Swamp, Folkston, Ga. The Middle and North Oconee Rivers were both sampled at two sites approximately 70 m apart, including a calm area stirred by eddy currents (upstream sites) and an area in the main flow of the river (downstream sites). In addition to periphyton samples, water samples were collected from these sites and from four additional sites; the additional sites were the Marine Institute Pond on Sapelo Island, Ga., Tuskegee Lake, Tuskegee, Ala., Wacissa Springs (sampled at the springhead), near Capps, Fla., and Muckalee Creek, Americus, Ga. These sites were sampled during the summers of 1984 and 1985.

Reagents. *p*-Cresol (purity, >99%) was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Filter-sterilized deionized water was used to make an aqueous stock by adding crystals of *p*-cresol dissolved in a few drops of 1 N NaOH. Aqueous stocks of NH_3NO_3 and K_2HPO_4 were made by using reagent grade chemicals (J. T. Baker Chemical Co., Phillipsburg, N.J.). The methylene chloride used was high-pressure liquid chromatography grade and was obtained from Burdick & Jackson Laboratories, Inc. The D-glucose, glycerol, and sodium bicarbonate (Fisher Scientific Co., Pittsburgh Pa.) and glycolic acid (Eastman Kodak Co., Rochester, N.Y.) used were reagent grade. The 3-(3,4-dichlorophenyl)-1,1-dimethylurea (ICN Pharmaceuticals Inc., Irvine, Calif., and K & K Laboratories, Inc., Plainview, N.Y.) used was technical grade. Amphotericin B was obtained from Sigma Chemical Co., St. Louis, Mo.

***p*-Cresol transformation experiments.** For estimating percentages of transformer bacteria, water samples from field sites were plated onto nutrient agar (BBL Microbiology Systems, Cockeysville, Md.) and incubated at 25°C for 48 h.

Because of the high temporal and spatial variability of the aufwuchs and periphyton, water samples were used in these studies as more homogeneous indicators of the presence of *p*-cresol-transforming bacteria. A total of 50 colonies representing each field site were transferred to nutrient agar slants and to 250-ml flasks containing 100 ml of nutrient broth (BBL) that was diluted 1:10 with distilled water. Colonies were chosen by picking most or all of the colonies on plates containing 50 or fewer colonies. The broth cultures were incubated at 20°C in a shaker-incubator at 180 rpm for 24 h, and then the cultures that were slightly cloudy from bacterial growth were amended with a sterile aqueous *p*-cresol stock to a concentration of approximately 300 μg/liter. This procedure lessened the probability of significant contamination of the cultures. The amended cultures were incubated at 20°C in the shaker-incubator for 1 week and then extracted for analyses of *p*-cresol. The method described above was chosen because it provides estimates of the concentrations of bacteria that are capable of transforming the parent compound in the presence of other nutrients rather than estimates of the concentrations of only those bacteria that are capable of using the compound as a sole carbon or energy source or mineralizing it to CO_2.

With few exceptions, cultures either transformed all or none of the *p*-cresol. Nontransforming cultures that were incubated for an additional 2 weeks rarely transformed *p*-cresol; therefore, incubation for 1 week was considered satisfactory for indicating the presence of populations that were capable of transforming the test chemical. Percentages of transformers were calculated by dividing the number of cultures that transformed *p*-cresol by the number of isolates that grew up in broth cultures and multiplying by 100. Nutrient agar slants corresponding to cultures that transformed *p*-cresol in broth cultures were transferred weekly for use in subsequent pure culture studies. However, most cultures did not survive more than one transfer. Only one culture, which was isolated from Wacissa Springs, was transferred successfully for several months, but it finally lost its ability to transform the test chemical. Based on previously published descriptions (3), this isolate was identified as a *Pseudomonas* species.

Experiments in which we used field-collected periphyton samples (see Table 1 and Fig. 1 through 3) were carried out by colonizing Teflon strips at each field site and incubating the strips at room temperature in beakers of water collected from the sites. Lag periods varied severalfold among the replicate periphyton samples tested at the field sites compared with samples stored and transported for 2 or 3 days. Therefore, lag periods were determined at field sites, except for samples that were collected within a 1-h transportation time from the laboratory. Experiments were carried out under a fluorescent light bank that provided 5.2 klx of illumination at the water surface. Light levels varied ±3% among beakers; therefore, beaker positions were varied among experiments to avoid systematic light effects. Strips were colonized for approximately 6 weeks during 1984 (see Table 1) and for approximately 1 year during 1984 and 1985 (see Fig. 1 through 3). Colonized strips (area, 516 cm^2) were fastened either in 4-liter beakers containing 3.8 liters (see Table 1) or in 2-liter beakers containing 1.8 liters (see Fig. 1 through 3). All beakers were stirred at 63 rpm with stainless steel paddle wheels as in previous studies (7). Using a sterile aqueous stock of *p*-cresol, we amended the beakers to a concentration of approximately 100 μg/liter and amended them at least one additional time. Because Teflon strips were destroyed at the Marine Institute Pond, we used grab samples of aufwuchs (floating mats and attached microbiota) at this site by adding approximately 4 g (wet weight) of aufwuchs per liter in 1,800 ml of site-collected water and stirring as in the periphyton studies. This procedure probably gave less reproducible results than the procedure in which replicate strips of colonized Teflon were used. Experiments in which we investigated the effects of carbon, nitrogen, and phosphorus on transformation lag periods were done by amending cultures with aqueous stock solutions and simultaneously amending with *p*-cresol. The effects of various amendments on bacterial growth were tested by determining viable plate counts for periphyton samples blended for 30 s with an electric blender. We also used aqueous stocks to amend some cultures to concentrations of 0.1 mg of 3-(3,4-dichlorophenyl)-1,1-dimethylurea per liter and 100 mg of amphotericin B per liter 1 h prior to amending with *p*-cresol. Using a Beckman Zeromatic model SS-3 pH meter, we determined pH values before and after water samples were amended, and no measurable effect on pH was observed.

Most transformation rate data indicated that there was some transformation of *p*-cresol from the time of the initial amendment; therefore, lag periods could not be determined based on the initial time of measurable transformation. Consequently, we used the first half-life as a measure of lag periods. The first half-life was defined as the time at which one-half of the initial *p*-cresol concentration had been lost from solution. Based on replicate periphyton-colonized strips from the two pond sites, the coefficient of variation for the first half-life was 2.1% (standard error, ±0.55%; $n = 12$).

Quantitative analyses. The *p*-cresol concentrations used for the experiment shown in Table 1 were determined by high-pressure liquid chromatography, using a Tracor high-pressure liquid chromatograph (Southern Analytical Inc., Oakwood, Ga.) equipped with a UV-VIS detector (279 nm) and a Zorbax CN column. For each sample, three extractions were made with methylene chloride, and the preparations were concentrated by using a steam bath. The extraction efficiency was 93%. For the experiments shown in Fig. 1 through 3, *p*-cresol concentrations were determined by gas-liquid chromatography, using a Tracor model 222 gas-liquid chromatograph equipped with a flame ionization detector and a 1-m column (internal diameter, 2 mm) packed with SP-1000 on 80/100-mesh Carbopak C (Supelco, Inc., Bellefonte, Pa.). Samples were extracted with a mixture containing 20 parts of sample to 1 part of methylene chloride. The extraction efficiency was 75%.

Nitrogen, phosphorus, and periphyton biomass (ash-free dry weight) concentrations were determined by using previously described procedures (18). The nitrogen concentrations represented the sums of the ammonia, nitrite, and nitrate concentrations. Most of the nitrogen was present as nitrate at all sites except the Okefenokee Swamp and Marine Institute Pond sites, where most of the nitrogen was present as ammonia. The other biomass estimates used included viable bacterial plate counts on nutrient agar incubated for 48 h at 25°C.

RESULTS AND DISCUSSION

Bacteria that were capable of transforming *p*-cresol were isolated from all of the field sites tested. Moreover, it was not uncommon that a large fraction of the bacteria isolated from a field site transformed *p*-cresol; samples from six of the eight sites tested contained 25% or more transformer bacteria. Similar results have been reported for xenobiotic chemicals, such as methyl parathion and diethyl phthalate

TABLE 1. Transformation lag periods and chemical data for field-collected periphyton samples grown in laboratory batch cultures

Site	N concn (mg/liter)[a]	P concn (mg/liter)[b]	Ash-free dry wt (mg/cm²)[c]	*p*-Cresol lag (h)[d]
River sites				
North Oconee (upstream)	0.38 ± 0.054	0.027 ± 0.009	3.4 ± 3.0	6.5 ± 0.26 (8)[e]
North Oconee (downstream)	0.39 ± 0.048	0.027 ± 0.012	1.1 ± 0.65	5.5 ± 0.35 (7)
Middle Oconee (upstream)	0.51 ± 0.025	0.092 ± 0.016	0.48 ± 0.01	7.1 ± 1.3 (7)
Middle Oconee (downstream)	0.64 ± 0.032	0.071 ± 0.024	0.05 ± 0.003	5.3 ± 0.62 (4)
Pond sites				
Chalet (inlet)	0.022 ± 0.018	0.020 ± 0.023	0.41 ± 0.06	47 ± 1.6 (8)
Chalet (outlet)	0.005 ± 0.003	0.013 ± 0.005	0.37 ± 0.05	36 ± 1.1 (8)
Two-Boat (inlet)	0.004 ± 0.001	0.009 ± 0.003	0.56 ± 0.02	>55 ± 2.8 (4)
Two-Boat (outlet)	0.015 ± 0.010	0.011 ± 0.007	0.42 ± 0.05	54 ± 2.3 (8)

[a] Mean total nitrogen concentration in water collected at the site ± standard error of the mean (n = 3).
[b] Mean PO_4 phosphorus concentration in water collected at the site ± standard error of the mean (n = 3).
[c] Mean ± standard error of the mean (n = 2).
[d] Mean first half-life ± standard error of the mean.
[e] The numbers in parentheses are numbers of batch cultures tested.

(7), suggesting that xenobiotic chemicals are transformed via systems which are used for naturally occurring substrates.

Using field-collected periphyton samples, we found that microbial samples collected from pond sites that were poor in dissolved N and P exhibited longer adaptation periods to *p*-cresol than samples collected from river sites, which had higher N and P concentrations (Table 1). Moreover, amending the pond samples with N and P to concentrations similar to the concentrations in the river samples caused shorter lag periods, which were similar to the lag periods of river samples (Fig. 1 and 2).

For Two-Boat Pond, which was moderately high in dissolved N and low in dissolved P (0.142 and 0.002 mg/liter, respectively), P amendments had a greater effect on the lag period than N amendments (Fig. 1). For the Okefenokee Swamp site, however, which was low in N and very high in P (0.041 and 2.2 mg/liter, respectively), only the addition of N shortened the lag period. Similarly, the Marine Institute Pond had only moderately high concentrations of both N and P (0.183 and 0.01 mg/liter, respectively), and amendments of both N and P shortened the lag period. All of the above-mentioned amendments consisted of amending the batch cultures to concentrations of 2.3 and 0.028 mg of additional N and P per liter, respectively. These concentrations of amendments were similar to the N and P concentrations determined for river samples that exhibited short lag periods.

The magnitude of the effect of limiting the nutrient concentration on the lag period was shown by adding various concentrations of P to batch cultures of periphyton-colonized strips (Fig. 2). Below a P concentration of about 0.01 mg/liter, the lag times were greatly lengthened by even small decreases in the initial P concentration; above a P concentration of approximately 0.15 mg/liter, the lag times

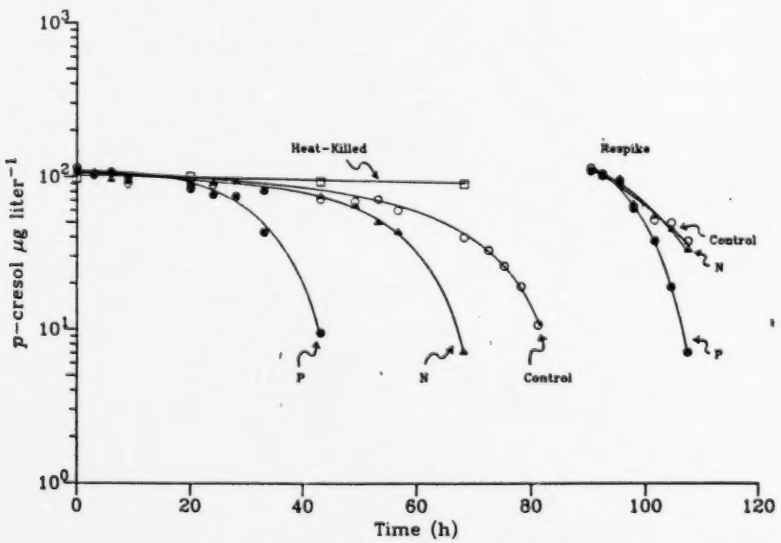

FIG. 1. Addition of 2.3 mg of nitrogen as NH_4NO_3 per liter and 0.028 mg of phosphorus as K_2HPO_4 per liter to periphyton samples amended with *p*-cresol. Periphyton samples were collected from Two-Boat Pond, which contained 0.14 and 0.002 mg of dissolved N and P per liter, respectively. The coefficient of variation among 12 replicate control (unamended with N and P) samples for adaptation periods based on initial half-life times was ±2%.

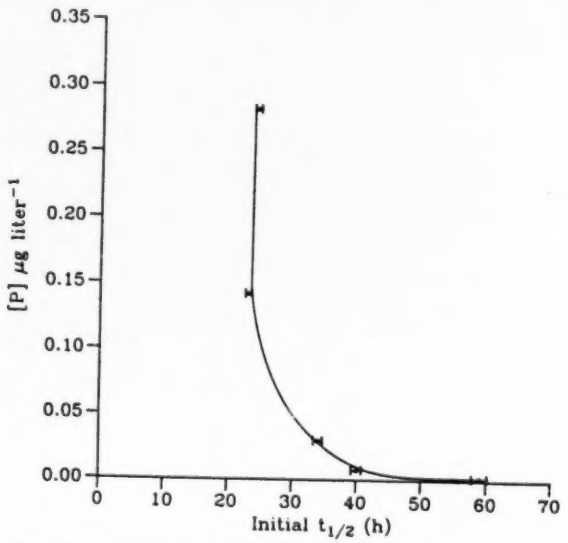

FIG. 2. Effect of phosphorus concentration (as K_2HPO_4) on adaptation times measured as initial half-lives ($t_{1/2}$) of *p*-cresol by using periphyton samples from Two-Boat Pond. The concentrations represent the total dissolved P, including 0.002 mg/liter that was present in the water from the field site. The bars indicate coefficients of variation.

were unaffected by increases in the initial P concentration. Therefore, for environmental modeling, it appears that it was possible to determine concentrations of commonly limiting nutrients below which adaptation lag periods would be related to limiting nutrient concentrations.

After untreated and amended cultures began to rapidly transform *p*-cresol, the cultures were amended again by using the same concentration of *p*-cresol as the initial amendment (Fig. 1). The transformation rates showed that cultures amended with N or P continued to transform *p*-cresol at slightly higher rates than untreated samples. Higher concentrations of *p*-cresol-transforming organisms that were present in periphyton surfaces amended with nutrients or higher transformation rates per cell in amended cultures compared with untreated cultures or both may have caused this small increase in transformation rates. Similarly, Horowitz and Atlas (6) reported increases in hydrocarbon-degrading bacteria and degradation rates after N and P additions in a flowthrough system.

The addition of 20 mg of carbon per liter as organic carbon sources did not shorten the lag periods for *p*-cresol transformation in periphyton samples from Two-Boat Pond (Fig. 3). Moreover, glycolic acid and glucose inhibited transformation like the inhibition of phenol transformation described by other researchers (14). In separate experiments, 3-(3,4-dichlorophenyl)-1,1-dimethylurea reduced the *p*-cresol transformation lag period by a factor of two, perhaps by preventing the photosynthetic production of glycolic acid or by being degraded and assimilated as a nitrogen source. Inorganic carbon also shortened the lag period (Fig. 3), probably as a result of increased availability of nitrogen provided by members of the *Rivulariaceae*, *Tolypothrix*, and other nitrogen-fixing cyanobacteria that appeared to be the predominant organisms in the samples. The same effect of inorganic carbon on lag periods was observed when we used Okefenokee Swamp samples dominated by *Anabaena*, *Chroococcus*, and *Oscillatoria* spp. Although increased nitrogen concentrations could not be detected in water samples, cyanobacteria were microscopically observed in close association with other bacteria that may have been able to scavenge excess fixed nitrogen before it could be dispersed in the water phase. Amphotericin B, which causes ammonia

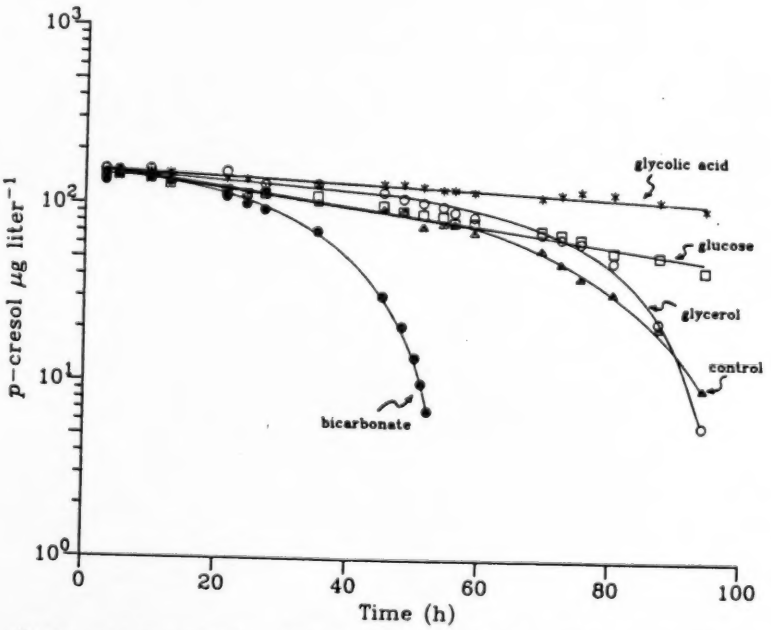

FIG. 3. Effect of 20 mg of carbon per liter as organic and inorganic amendments to periphyton samples from Two-Boat Pond amended with *p*-cresol.

and other essential nutrients to rapidly leach out of eucaryotes, also shortened *p*-cresol transformation lag periods by a factor of two in samples from Two-Boat Pond. Therefore, it is evident from these observations that complex microbial community interactions may sometimes significantly influence adaptation periods.

When our experimental concentrations of N, P, and HCO_3 were added to periphyton slurries, only P caused measurable increases in bacterial cell concentrations. Regardless of biomass concentration, however, the transformation rates for a given ratio of colonized surface area to volume at a given turbulence generally vary by less than a factor of five (7–9). In one study (8), for example, a 150-fold range in bacterial concentration (after nutrient additions to periphyton samples) caused only about a twofold variation in surface biofilm transformation rates of a herbicide. Therefore, the 100-fold or more change in *p*-cresol transformation rates observed during adaptation could not be explained by biofilm growth. If biomass concentrations per unit surface area affected lag periods, adaptation times should have been decreased by the addition of organic carbon, unless only non-*p*-cresol tranformers grew when the additional carbon was added. Because a high proportion of the bacteria isolated from the field sites transformed *p*-cresol, it is unlikely that selective growth of nontransformers occurred.

Although we found that lag periods were related to limiting nutrient concentrations, which may provide a basis for predicting lag times, our data failed to elucidate the specific mechanism responsible for lag periods. Limiting nutrients may (i) limit the production of *p*-cresol-transforming enzymes, (ii) limit the mRNA synthesis required to induce *p*-cresol-transforming enzymes, (iii) limit the production of enzymes that are capable of removing compounds that repress synthesis of *p*-cresol-transforming enzymes, (iv) limit the synthesis of plasmids that are involved in *p*-cresol transformation, or (v) cause any combination of the above or additional, unexpected effects.

The significance of the relationship between limiting nutrients and adaptation may be inconsequential for some environmental systems. For soil, groundwater, and marine systems it may never matter whether the microbial communities begin transforming a xenobiotic pollutant immediately or 2 weeks later. For surface freshwater systems, especially rivers and streams, however, adaptation periods on the order of days can delay abatement of the pollutant long enough for it to reach municipal water supplies or other areas of critical concern.

Should it be shown to be useful to correlate adaptation periods with a limiting nutrient, the question arises as to what the limiting nutrients for various ecosystems are. The concept of a single limiting nutrient is a concept that serves well for agricultural systems pertaining to the production of a particular crop; however, environmental systems are comprised-of innumerable organisms, each having its own peculiar nutrient requirements. Therefore, a number of nutrients may be limiting at any given time for various components of the system. In general, however, N is considered to be the most common limiting nutrient in marine systems (10, 15), and P is most commonly limiting in freshwater systems, such as lakes (4, 5, 11–13, 15). Because pollutant transformations are carried out primarily by bacteria and fungi, which have higher N demands than primary producers, upon which the studies described above were based, N may still frequently limit the heterotrophic activities associated with pollutant transformations in freshwater ecosystems. Even in freshwater communities of primary producers, N can be considered to have a significant secondary effect on net production (16). Therefore, it is evident that extensive studies involving numerous test chemicals and field-collected samples of microbiota (or in situ field studies) will have to be done to determine whether N or P concentrations are a useful basis for predicting the adaptation time for biotransformation rates of pollutants in the environment.

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